10/735,423
Claims For ODP

CLAIMS

What is claimed is:

1. A method for cloning a non-human mammal through a nuclear transfer process comprising:

(i) obtaining desired differentiated mammalian cells to be used as a source of donor nuclei;

(ii) obtaining at least one oocyte from a mammal of the same species as the cells which are the source of donor nuclei;

(iii) enucleating said at least one oocyte;

(iv) transferring the desired differentiated cell or cell nucleus into the enucleated oocyte;

(v) simultaneously fusing and activating the cell couplet to form a first transgenic embryo;

(vi) culturing said first transgenic embryo(es) until it reaches at least the 2-cell developmental stage; and

(vii) using at least one of the cells of said first transgenic embryo as a donor cell for the production of a transgenic animal through at least a second round of nuclear transfer so as to produce a second transgenic embryo; and

2. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from mesoderm.

3. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from endoderm.

4. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from ectoderm.

5. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from fetal somatic tissue.

6. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from fetal somatic cells.

7. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from a fibroblast.

8. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from an ungulate.

9. The method of either claims 1 or 8, wherein said donor cell or donor cell nucleus is from an ungulate selected from the group consisting of bovine, ovine, porcine, equine, caprine and buffalo.

10. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from an adult non-human mammalian somatic cell.

11. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells.

12. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is from an organ selected from the group consisting of skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organ, bladder, kidney and urethra.

13. The method of claim 1, wherein said at least one oocyte is matured *in vivo* prior to enucleation.

14. The method of claim 1, wherein said at least one oocyte is matured *in vitro* prior to enucleation.

15. The method of claim 1, wherein said non-human mammal is a rodent.

16. The method of claim 1, wherein said donor differentiated mammalian cell to be used as a source of donor nuclei or donor cell nucleus is a non-quiescent somatic cell or a nucleus isolated from said non-quiescent somatic cell.

17. The method of either claims 1 or 8, wherein the fetus develops into an offspring.

18. The method of claim 1, wherein said at least one oocyte is enucleated about 10 to 60 hours after initiation of *in vitro* maturation.

19. The method of claim 1, wherein a desired gene is inserted, removed or modified in said differentiated mammalian cell or cell nucleus prior to insertion of said differentiated mammalian cell or cell nucleus into said enucleated oocyte.

20. The resultant offspring of the methods of claims 1 or 19.

21. The resultant offspring of claim 19 further comprising wherein the offspring created as a result of said nuclear transfer procedure is chimeric.

22. The method of claim 1, wherein cytocholasin-B is used in the cloning protocol.

23. The method of claim 1, wherein cytocholasin-B is not used in the cloning protocol.

24. The method of claim 1, wherein said differentiated mammalian cells to be used as a source of donor nuclei is from an in vitro matured oocyte.

25. The method of claim 24 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at most at the 40-cell stage of development.

26. The method of claim 24 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 32-cell stage of development.

27. The method of claim 24 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 16-cell stage of development.

28. The method of claim 24 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 8-cell stage of development.

29. The method of claim 1, wherein said differentiated mammalian cells to be used as a source of donor nuclei is from an in vivo matured oocyte.

30. The method of claim 29 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at most at the 40-cell stage of development.

31. The method of claim 29 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 32-cell stage of development.

32. The method of claim 29 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 16-cell stage of development.

33. The method of claim 29 wherein the cells of said first transgenic embryo are utilized for the generation of at least one said second transgenic embryo when said first transgenic embryo is at the 8-cell stage of development.

34. The method of claim 1, further comprising transferring said second transgenic embryo into a host mammal such that the embryo develops into a fetus.

35. The resultant offspring of the methods of claims 24 or 29.

542

stage of development. As the animals develop and grow the protein product or specific developmental change engineered into the animal becomes apparent.

[005] At present the techniques available for the generation of transgenic domestic animals are inefficient and time-consuming typically producing a very low percentage of viable embryos. During the development of a transgene, DNA sequences are typically inserted at random, which can cause a variety of problems. The first of these problems is insertional inactivation, which is inactivation of an essential gene due to disruption of the coding or regulatory sequences by the incoming DNA. Another problem is that the transgene may either be not incorporated at all, or incorporated but not expressed. A further problem is the possibility of inaccurate regulation due to positional effects. This refers to the variability in the level of gene expression and the accuracy of gene regulation between different founder animals produced with the same transgenic constructs. Thus, it is not uncommon to generate a large number of founder animals and often confirm that less than 5% express the transgene in a manner that warrants the maintenance of the transgenic line.

[006] Additionally, the efficiency of generating transgenic domestic animals is low, with efficiencies of 1 in 100 offspring generated being transgenic not uncommon (Wall, 1997). As a result the cost associated with generation of transgenic animals can be as much as 250-500 thousand dollars per expressing animal (Wall, 1997).

[007] Prior art methods have typically used embryonic cell types in cloning procedures. This includes work by Campbell et al (Nature, 1996) and Stice et al (Biol. Reprod., 1996). In both of those studies, embryonic cell lines were derived from embryos of less than 10 days of gestation. In both studies, the cells were maintained on a feeder layer to prevent overt differentiation of the donor cell to be used in the cloning procedure. The present invention uses differentiated cells. It is considered that embryonic cell types could also be used in the methods of the current invention along with cloned embryos starting with differentiated donor nuclei.

[008] Thus, according to the present invention, multiplication of superior genotypes of mammals, including caprines, is possible. This will allow the multiplication of adult animals with proven genetic superiority or other desirable traits. Progress will be accelerated, for example, in many important mammalian species including goats, rodents, cows and rabbits. By the present invention, there are potentially billions of fetal or adult cells that can be harvested and used in the cloning procedure. This will potentially result in many identical offspring in a short period.

tissue types (Zou *et al.*, 2001 and Wells *et al.*, 1999), as well as embryonic (Yang *et al.*, 1992; Bondioli *et al.*, 1990; and Meng *et al.*, 1997), have also been reported.

[0023] According to the current invention, the use of recombinant somatic cell lines for nuclear transfer, and improving this procedures efficiency by increasing the number of available cells through the use of "re-cloned" embryos, not only allows the introduction of transgenes by traditional transfection methods into more transgenic animals but also increases the efficiency of transgenic animal production substantially and improves cellular reprogramming to allow normal and healthy development of transgenic embryos allowed to develop into animals.

[0024] Through the methodology and system employed in the current invention transgenic animals, goats, were generated by somatic cell nuclear transfer and were shown to be capable of producing a target therapeutic protein in the milk of a cloned animal.

[0025] According to a preferred embodiment of the current invention, embryos resulting from somatic cell NT (nuclear transfer) at various cell stages, including the 2-,40-, 8-, 16-, 32-cell, Morula, and Blastocyst cell stages can be used as cell donors to produce transgenic animals by a second round of NT (re-cloning).

[0026] In an additional embodiment of the current invention, embryos resulting from the use of in vitro matured oocytes for NT at various cell stages, including the 2-, 40-, 8-, 16-, 32-cell, Morula, and Blastocyst cell stages can be used as cell donors to produce transgenic animals through a second round of NT.

[0027] In an additional embodiment of the current invention, embryos resulting from the use of in vivo matured oocytes for NT at various cell stages, including the 2-, 40-, 8-, 16-, 32-cell, Morula, and Blastocyst cell stages can be used as cell donors to produce transgenic animals through a second round of NT.

[0028] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.